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Title: Ionizing Radiation Sensitivity of *Listeria monocytogenes* ATCC 49594 and *Listeria innocua* ATCC 51742 Inoculated on Endive (*Cichorium endiva*)

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Ionizing Radiation Sensitivity of *Listeria monocytogenes* ATCC 49594 and *Listeria innocua* ATCC 51742 Inoculated on Endive (*Cichorium endiva*)[†]

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ABSTRACT

Ionizing radiation inactivates the pathogenic bacteria that can contaminate leafy green vegetables. Leaf pieces and leaf homogenate of endive (*Cichorium endiva*) were inoculated with the pathogen *Listeria monocytogenes* (ATCC 49594) or *Listeria innocua* (ATCC 51742), a nonpathogenic surrogate bacterium. The radiation sensitivity of the two strains was similar, although *L. innocua* was more sensitive to the type of suspending leaf preparation. During refrigerated storage after irradiation, the population of *L. monocytogenes* on inoculated endive was briefly suppressed by 0.42 kilogray (kGy), a dose calibrated to achieve a 99% reduction. However, the pathogen regrew after 5 days until it exceeded the bacterial levels on the control after 19 days in storage. Treatment with 0.84 kGy, equivalent to a 99.99% reduction, suppressed *L. monocytogenes* throughout refrigerated storage. Doses up to 1.0 kGy had no significant effect on the color of endive leaf material, regardless of whether taken from the leaf edge or the leaf midrib. The texture of leaf edge material was unaffected by doses up to 1.0 kGy, whereas the maximum dose tolerated by leaf midrib material was 0.8 kGy. These results show that endive leaves may be treated with doses sufficient to achieve at least a 99.99% reduction of *L. monocytogenes* with little or no impact on the product's texture or color.

Fresh produce has been associated with numerous outbreaks of foodborne illness in North America in recent years (2). Salad vegetables, including fresh-cut lettuce, can be a source of pathogens such as *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* spp. (17). The possibility of contaminated food products increases with additional handling and processing steps (3). Treatment of vegetables with ionizing radiation can effectively eliminate pathogenic bacteria (13). The radiation sensitivity of pathogenic bacteria can be influenced by product composition (12).

L. monocytogenes is a foodborne bacterium responsible for numerous foodborne illness outbreaks and product recalls (4). *L. monocytogenes* can be a contaminant in a variety of food products, including vegetables (1, 2). The mortality frequency for listeriosis patients is approximately 20%; *L. monocytogenes* is subject to zero-tolerance regulation in ready-to-eat meat products in the United States (7, 19). In environments where pathogens may not be used, the nonpathogenic bacterium *Listeria innocua* is commonly used in decontamination studies as a surrogate for *L. monocytogenes* (16). Surrogate organisms can yield important information about how their associated pathogen might respond to a given antimicrobial treatment. However, *L. innocua* has been shown to differ in from *L. monocytogenes*

with regard to attachment behavior (8); this has implications for intervention studies that address the formation of biofilms, the removal of biofilms, or both in which *L. monocytogenes* may be a participant. Thus, a given nonpathogenic surrogate may be a good model with regard to one parameter (e.g., sensitivity to an antimicrobial agent or process) but a poor model with regard to another (e.g., attachment). Different species within a given bacterial genus can have significantly different sensitivities to ionizing radiation, even when treated with the same product under the same conditions (11).

The objectives of this study were to determine (i) the radiation sensitivity of a pathogen, *L. monocytogenes*, and a commonly used surrogate, *L. innocua*, when inoculated onto endive (*Cichorium endiva*), a leafy salad vegetable; (ii) the survival and potential for regrowth of *L. monocytogenes* on irradiated, stored endive; and (iii) the effect of efficacious doses of radiation on the texture and color of endive leaves.

MATERIALS AND METHODS

Products. Whole heads of endive were purchased from local markets on the day of each experiment. The outer leaves were discarded. For microbiological testing, cut leaf pieces were prepared from the entire head. The basal portion of the head was removed approximately 5 cm from the end. The leaves were sliced as a group into pieces weighing approximately 0.5 g. Fresh produce typically carried a native microbial load (10); before use in the experiments, the leaf material was surface sanitized with a 300-ppm sodium hypochlorite solution according to the method of Niemira et al. (12). Briefly, the leaf pieces were gently agitated

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† Mention of brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others of similar nature not mentioned.

in the room-temperature sanitizing solution for 3 min, thoroughly rinsed in distilled water, and spun in a sterile salad spinner-type centrifuge to remove excess surface water (Oxo International, New York, N.Y.). This design of salad spinner incorporates a container base that captures all of the water removed from the leaf surface and prevents the formation of aerosolized droplets. The microflora of sanitized leaf material was measured using a surface wash with Butterfield's phosphate buffer (BPB; Applied Research Institute, Newtown, Conn.), serial dilution, pour plating with tryptic soy agar (TSA; Difco, Detroit, Mich.), and incubation at 37°C for 24 h. The postsanitization population was less than 20 CFU/g of leaf tissue.

Homogenized leaf tissue was used as a model solution to determine the effect of internal leaf chemistries on the radiation sensitivity of internalized bacterial populations (12). Homogenized leaf suspensions were prepared using sanitized cut leaf tissue. A sample of 45 g of leaf material was placed into a sterile Osterizer-style blender jar (Thomas Scientific, Swedesboro, N.J.) with 180 ml of sterile BPB. This was blended at high speed using a laboratory-grade Osterizer-style blender (Thomas Scientific) for 5 s to completely homogenize the leaf material. The homogenate was poured through four layers of sterile cheesecloth into a sterile beaker.

Microorganisms. A representative strain of *L. monocytogenes* (ATCC 49594; American Type Culture Collection, Manassas, Va.) and a nonpathogenic strain of *L. innocua* (ATCC 51742) were maintained on 50% glycerol at -70°C. A frozen culture was regrown in tryptic soy broth (TSB; Difco) for 16 h at 37°C with agitation and streaked onto Palcam agar (Difco). This was incubated at 37°C for 48 h to form single colonies. These colonies were used to inoculate fresh TSB for each experiment, grown for 16 h at 37°C with agitation. The cell density of the starting inoculum was determined by serial dilution with sterile BPB and pour plating with TSA. The cell density was typically 10⁹ CFU/ml.

To determine the amount of radiation necessary to eliminate 90% of the population (D_{10}), leaf material was inoculated before irradiation. The homogenized leaf suspension (99 ml) was inoculated with 1 ml of either *L. monocytogenes* or *L. innocua* culture. Aliquots (5 ml) of the inoculated suspension were dispensed into sterile glass tubes. One tube was used per culture per dose. The experiment was performed three times.

Cut leaf pieces were inoculated separately according to the method of Niemira et al. (12). Because of the potential for aerosolization of the inoculum associated with this method, all material preparation involving the microorganisms was conducted in a biological airflow hood under strict adherence to worker safety guidelines. Sanitized leaf pieces were transferred to a sterile glass inoculation dish (22 by 33 by 5 cm), and 1,000 ml of the working inoculum of either *L. monocytogenes* or *L. innocua* was added. The material was agitated gently for 120 s to completely submerge each piece and then transferred to a sterile salad spinner-type centrifuge (Oxo). The material was spun twice to remove excess inoculum from the surface of the leaf pieces. Samples (45 g) of each lettuce type were placed in no. 400 stomacher bags (Tekmar, Inc., Cincinnati, Ohio). The samples were refrigerated (4°C) until irradiation, typically 30 to 60 min. The experiment was performed three times.

In separate studies to determine the survival and recovery of *L. monocytogenes* on irradiated endive during refrigerated storage, cut leaf pieces were inoculated with *L. monocytogenes* and bagged as described. Multiple bagged samples were prepared for a storage

study of 19 days' duration. The experiment was performed three times.

Irradiation. The samples were irradiated using a Lockheed-Georgia (Marietta, Ga.) cesium-137 self-contained gamma radiation source with a dose rate of 0.098 kilogray (kGy)/min. The dose rate for this irradiator was previously established using National Institutes of Standards and Technology protocols and dosimeters. Corrections for the decay rate of the cesium-137 source are made weekly. The samples were oriented with a similar geometry in the center of the radiation field for all treatments. The source pencils for this irradiator are in an annular array, which ensures evenness of exposure. The temperature of the samples was held at 2°C during irradiation by the injection of gas-phase liquid nitrogen. The temperature was monitored during irradiation with calibrated thermocouples. Alanine pellets (Bruker, Inc., Billerica, Mass.) were used for dosimetry. The pellets were read on a Bruker EMS 104 electron paramagnetic resonance analyzer and compared with a previously determined standard curve. The delivered dose, as determined by electron paramagnetic resonance dosimetry, was typically within 5% of the nominal dose.

For the determination of decimal reduction times (D_{10} -values), the inoculated samples of homogenized leaf tissue were treated with 0.0 (control), 0.2, 0.4, 0.6, 0.8, or 1.0 kGy. Inoculated cut leaf pieces were treated with 0.0 (control), 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, or 1.0 kGy. For the storage study, the inoculated cut leaf pieces were treated with 0.0 (control), 0.42, or 0.84 kGy. These doses were selected to achieve a 0-, 2-, or 4-log₁₀ reduction (approximately), based on the D_{10} -values obtained for *L. monocytogenes*. The samples for the three replications of each study were irradiated concurrently.

Sampling. After irradiation, the samples were returned to refrigerated storage (2°C) until microbiological sampling, typically 30 to 60 min. For determination of D_{10} -values, aliquots (1 ml) of irradiated leaf homogenates were serially diluted with sterile BPB. Pour plating with TSA was used to determine the surviving bacterial population. Three pour plates per dilution were incubated for 24 h at 37°C and counted with an automatic plate counter. In the case of irradiated cut leaf pieces, sterile BPB (180 ml) was added to the stomacher bag and agitated for 60 s. A 1-ml sample was withdrawn for serial dilution with sterile BPB. The samples were diluted, pour plated with TSA, and incubated as described.

The data were normalized against the control and plotted as the log₁₀ reduction using the nominal doses. The slopes of the individual survivor curves were calculated with linear regression using a computer graphics program (SigmaPlot 5.0, SPSS Inc., Chicago, Ill.). The D_{10} -value for *L. monocytogenes* and *L. innocua* on each leaf preparation was calculated by taking the negative reciprocal of the survivor curve slope (QuattroPro, Corel Corp., Ontario, Canada). The significance of differences between the regression lines was determined using analysis of covariance (Excel, Microsoft Corp., Redmond, Wash.).

For the storage study, the irradiated leaf pieces were stored in air at 2°C until sampling. Samples were collected immediately after irradiation (day 0) and on days 2, 5, 14, and 19. The samples were surface washed with BPB, serially diluted with BPB, and pour plated with TSA as described. Simultaneous samples were plated on Palcam and TSA to distinguish the regrowth of *L. monocytogenes* from the regrowth of other aerobes. For key sampling times, analysis of variance (ANOVA) was used to evaluate the difference among the dose levels (SigmaStat; SPSS).

Sensory properties. Cut leaf pieces of endive were prepared from heads of endive purchased fresh from local markets. The

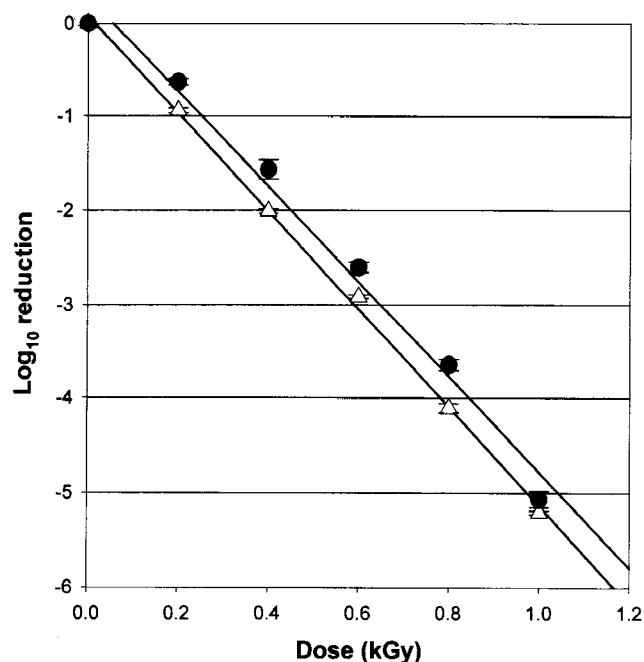


FIGURE 1. Response of *Listeria monocytogenes* (circle) and *L. innocua* (triangle) to irradiation in inoculated endive leaf homogenate. Lines are linear regression, and error bars indicate standard error ($n = 9$).

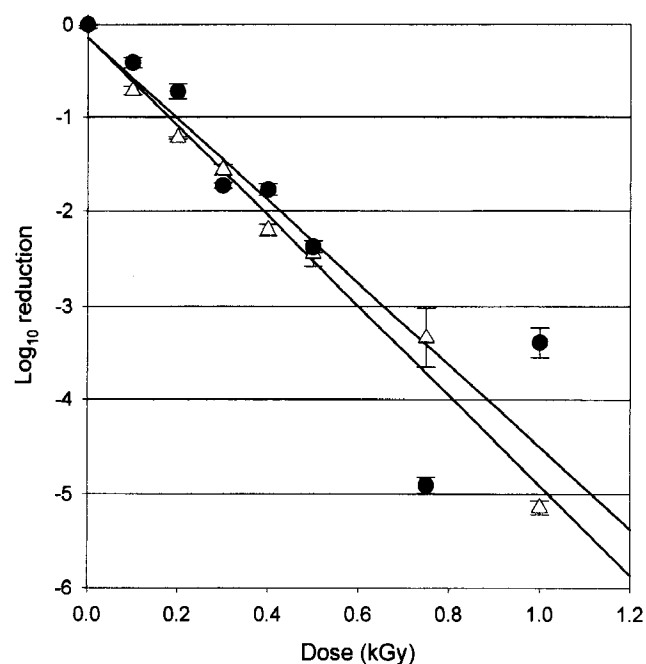


FIGURE 2. Response of *Listeria monocytogenes* (circle) and *L. innocua* (triangle) to irradiation on inoculated endive leaf pieces. Lines are linear regression, and error bars indicate standard error ($n = 9$).

outer leaves and the base of the heads were removed as described above. The proximal portions of the leaves, consisting primarily of leaf midrib tissue, were separated en masse from the distal portions of the leaves, consisting primarily of leaf edge tissue. These were separately cut into pieces, sanitized, rinsed, and spun dry as described above. Five samples of each type of leaf tissue were bagged for irradiation, 10 g per sample. The samples were treated with 0.0 (control), 0.2, 0.4, 0.6, 0.8, or 1.0 kGy, as described, and held at 2°C until sampling, typically 90 to 120 min. The study was performed three times, with samples irradiated concurrently. Data were pooled and analyzed with ANOVA as described.

Color. Color values were taken with a Hunter Laboratory (Reston, Va.) Miniscan XE meter to determine the brightness (L-value), greenness/redness (a-value), and blueness/yellowness (b-value) of the material. The meter was calibrated using white and black standard tiles. An illuminant D65, a 10° standard observer, and a 2.5-cm port/viewing area were used.

Texture. The maximum shear strength of the leaf sections was measured with a TA.XT2i texture analyzer running the TextureExpert version 1.22 software package (Texture Technologies, Scarsdale, N.Y.) using a TA-91 Kramer Shear Press with five blades.

RESULTS

D_{10} -values. Irradiation effectively reduced the population of *L. monocytogenes* and *L. innocua* in leaf homogenates (Fig. 1) and on cut leaf pieces (Fig. 2). The D_{10} -values obtained did not differ significantly ($P < 0.05$) between *L. monocytogenes* and *L. innocua* on either leaf homogenates or leaf pieces. However, the D_{10} -value for *L. innocua* was significantly ($P < 0.05$) lower on leaf homogenates than on leaf pieces, whereas the D_{10} -value for

L. monocytogenes was not sensitive to leaf preparation method (Table 1).

Storage and regrowth. Irradiation with either 0.42 or 0.84 kGy reduced the initial population of *L. monocytogenes* on endive pieces by 2.6 or 4.0 log₁₀ factors relative to the untreated control, which was close to the levels of reduction predicted by the calculated D_{10} -value (Fig. 3A, Palcam agar). The *L. monocytogenes* population was slightly reduced on the untreated controls after 2 days of refrigerated storage but remained stable thereafter throughout the 19 days of the study. After irradiation at 0.42 kGy, the population similarly declined slightly for 5 days and then rebounded and increased at 14 days. At the final sampling time, 19 days, the population of *L. monocytogenes* was slightly (0.4 log₁₀ unit) greater than on the control, a statistically significant difference ($P < 0.05$). After irradiation at 0.84 kGy, the levels of *L. monocytogenes* increased slightly during storage, although the population remained

TABLE 1. Radiation D_{10} -values of *Listeria monocytogenes* and *L. innocua* inoculated onto endive pieces and endive leaf homogenate

	Endive homogenate	Cut leaf pieces	
<i>L. monocytogenes</i>	0.20 ± 0.01 ^a	0.21 ± 0.01	NSD ^b
<i>L. innocua</i>	0.19 ± 0.01	0.22 ± 0.01	$P < 0.05$
	NSD	NSD	

^a Numbers are D_{10} -values plus or minus the standard error, in kGy.

^b For each inoculum type and for each leaf preparation, the results of analysis of covariance are shown, either as a P value or as "NSD" (i.e., no significant difference).

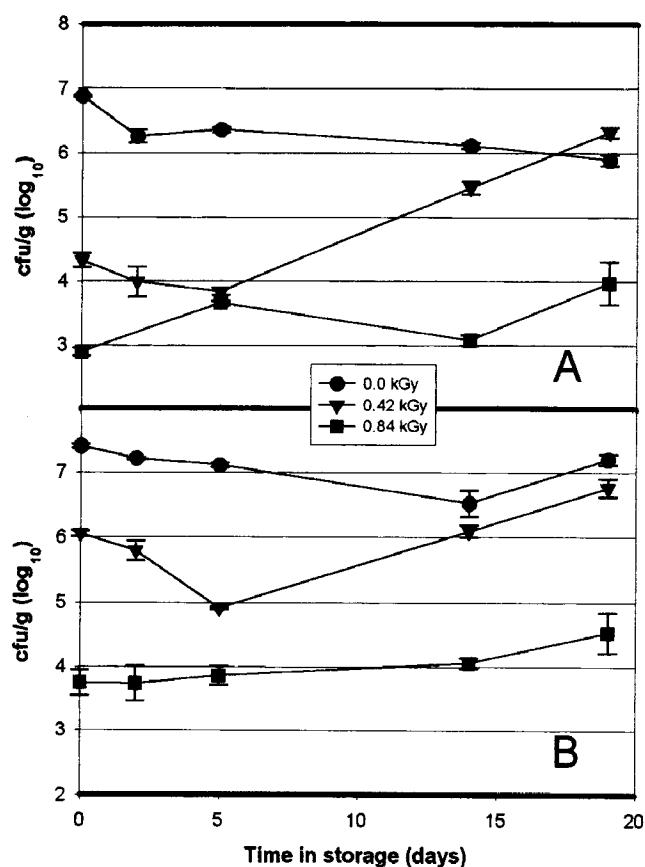


FIGURE 3. Survival and regrowth during refrigerated storage of *Listeria monocytogenes* on irradiated endive leaf pieces. Graphs represent growth on Palcam agar (A) or tryptic soy agar (B). Error bars indicate standard error ($n = 9$ per sampling time).

significantly lower than that of the control throughout the study.

The total aerobic plate counts obtained after incubation at 37°C were generally 0.5 to 1.0 \log_{10} units greater than counts on Palcam agar at the comparable dose–time combinations (Fig. 3B, TSA), indicating that *L. monocytogenes* was the dominant member of the microbial community. The behavior of the total aerobic plate counts in response to irradiation was generally similar to that of the *L. monocytogenes* population. The total aerobic plate counts increased on samples treated with 0.42 kGy until, at days 14 and 19,

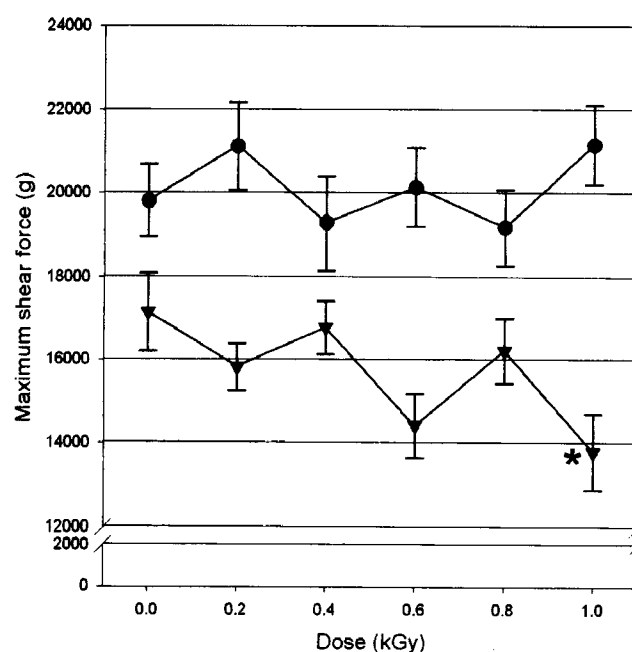


FIGURE 4. Texture of irradiated endive leaf edge segments (circle) or leaf midrib segments (triangle). For each type of leaf segment, doses denoted with an asterisk (*) are significantly ($P < 0.05$) different from their respective control by analysis of variance (ANOVA). Error bars indicate standard error ($n = 15$).

they were not significantly different from the controls. The total aerobic plate count was reduced after irradiation at 0.84 kGy, but by days 14 and 19, the counts were significantly higher than immediately after treatment (Fig. 3B).

Color. Irradiation up to 1.0 kGy had no significant effect on the color of leaf tissue (Table 2). Material taken from the leaf edge was generally darker and greener than material taken from the leaf midrib.

Texture For leaf material taken from the leaf edge, irradiation doses up to 1.0 kGy had no effect on texture, with the maximum shear force obtained at each dose not being statistically different (ANOVA, $P < 0.05$) from that of the control (Fig. 4). Material taken from the leaf midrib was similarly insensitive to doses up to 0.8 kGy; however, at the highest dose examined, 1.0 kGy, the maximum shear

TABLE 2. Color parameters of irradiated endive pieces

Dose (kGy)	Leaf edge sections			Leaf midrib sections		
	L ^a	a ^b	b ^c	L	a	b
0.0	37.47 A ^d	−9.13 A	25.89 A	57.35 A	−5.59 A	25.23 A
0.2	39.05 A	−8.79 A	25.06 A	53.57 A	−6.44 A	27.32 A
0.4	38.22 A	−8.57 A	24.89 A	53.39 A	−6.10 A	24.86 A
0.6	37.70 A	−9.04 A	25.45 A	55.14 A	−5.72 A	24.80 A
0.8	37.13 A	−8.52 A	23.85 A	56.91 A	−5.66 A	26.21 A
1.0	37.51 A	−8.91 A	24.42 A	55.02 A	−6.09 A	25.42 A

^a Brightness (“L”): 0 = white, 100 = black.

^b Green/Red (“a”): negative “a” values indicate greenness; positive “a” values indicate redness.

^c Blue/Yellow (“b”): negative “b” values indicate blueness; positive “b” values indicate yellowness.

^d For a given product, dose–temperature combinations followed by the same letter are not significantly different ($P < 0.05$, analysis of variance, Tukey test).

force was significantly less (ANOVA, $P < 0.05$) than that of the control (Fig. 4).

DISCUSSION

Ionizing radiation was effective in reducing the population viability of both *L. monocytogenes* and *L. innocua* on endive leaf preparations. Bacterial sensitivity to radiation can vary widely for the same pathogen on different food substrates (12, 18) or among different strains of a given pathogen, even when tested under identical conditions of processing and food milieu (11, 15). The evaluation of the radiation resistance response of a single isolate eliminates variability; however, the results obtained are necessarily a description of the behavior only of the isolate examined. Although data for the radiation D_{10} -value of *L. innocua* are lacking, the D_{10} -value for *L. monocytogenes* has been reported to range from 0.27 to 0.77 kGy, depending on the food substrate (9). The D_{10} -values obtained in this study are consistent with the lower end of this range (Table 1).

In this study, the responses of the strains of *L. monocytogenes* and *L. innocua* to irradiation did not differ from each other on either cut leaf pieces or leaf homogenates. These results suggest that, on this food substrate, *L. innocua* can be an effective surrogate for *L. monocytogenes*. As with any model, a surrogate bacteria should adequately mimic the behavior of the target pathogen, specifically with regard to the parameter being tested. Some strains of *L. innocua* are known to differ significantly from *L. monocytogenes* with regard to attachment (8). Surrogates should therefore not be expected to be a universal substitute, with similarity of response to one testing parameter implying similarity of response to another testing parameter.

Relatively subtle differences in the underlying substrate can significantly influence the radiation sensitivity of inoculated bacteria (12). In this study, the radiation D_{10} -value of *L. monocytogenes* was insensitive to changes in leaf tissue preparation, i.e., a liquid leaf homogenate suspension versus surface inoculation. *L. innocua*, in contrast, was significantly more sensitive to irradiation in leaf homogenate than on the leaf surface. Niemira et al. (12) found that suspension in a lettuce leaf homogenate as opposed to surface inoculation on leaf pieces rendered *E. coli* O157:H7 either significantly more or significantly less sensitive to ionizing radiation, depending on the type of lettuce being tested. This influence of substrate has been demonstrated with a variety of meat, fruit, and vegetable products (10, 14, 18). The mechanisms responsible for the influence of food substrate on radiation sensitivity of associated bacteria are not fully understood but are generally thought to be related to the substrate's ability to protect the bacteria from the oxygen and hydroxyl radicals formed during the irradiation process (5, 6). The leaf homogenate is intended as a simplified model for the type of chemistries that an internalized bacterial population may encounter, albeit a model with acknowledged limitations (12). The chemically complex leaf homogenates are a model for the type of chemistries that bacteria may be surrounded with following internalization. Internalized bacteria inhabit the intracellular

spaces between cells of leaf tissue (10). Given that the homogenate combines chemistries from the distinct anatomic regions of the leaf (intracellular fluid, cytoplasm, vacuoles, etc.), it is not a completely accurate model; however, it does provide a basic indication of the type of influence that internal leaf chemistries may have on the action of ionizing radiation (12). The results of this study suggest that *L. innocua* that is internalized in a leaf may be somewhat more sensitive than surface-associated contamination. It should be noted that the dynamics and growth of bacterial populations are influenced by incubation conditions and that additional studies that use different incubation temperatures, atmospheres, etc., will further elucidate the processes involved. The as-yet incomplete understanding of the influence of substrate on bacterial radiation sensitivity suggests that the behavior of a surrogate must be adequately validated, not only with the process being tested, but also on the food substrate of interest.

On stored endive leaf material, inoculated *L. monocytogenes* remained on the nonirradiated control material at levels approximately equal to that of the initial inoculation. A pretreatment with 0.42 kGy reduced the population of *L. monocytogenes* by 2.6 log₁₀ units. During refrigerated storage, however, *L. monocytogenes* regrew until it was equal to or greater than the control. A dose of 0.84 kGy provided a greater initial reduction and resulted in a suppression of *L. monocytogenes* throughout storage. The regrowth of *L. monocytogenes* on endive after irradiation at 0.42 kGy, as demonstrated, is similar to the results obtained by Prakash et al. (13) in a study of *L. monocytogenes*-inoculated celery. In that work, a dose of 0.5 kGy caused an initial 2.1-log₁₀ reduction from the control levels, followed by a regrowth during 20 days of refrigerated storage. Also in that study, a dose of 1.0 kGy was sufficient to cause a reduction in the *L. monocytogenes* population that lasted throughout storage. Low radiation doses, i.e., doses roughly equivalent to 2 D_{10} units, therefore provide a transitory reduction of *L. monocytogenes* populations. Because *L. monocytogenes* is able to conduct injury repair and grow at refrigeration temperatures, a higher initial radiation dose equivalent to 4 or more D_{10} units is required to effect a more complete elimination and avoid the possibility of regrowth of *L. monocytogenes* after an inadequate treatment.

Endive leaf pieces were generally insensitive to changes in color or texture after antimicrobially efficacious radiation doses. Doses equivalent to a 4.8-log₁₀ reduction had no significant impact on the color of endive leaf pieces, regardless of the part of the leaf sampled. Leaf edge pieces showed no significant change in texture at any dose tested, and leaf midrib pieces were similarly unaffected at doses up to 0.8 kGy. These results indicate that preparations of endive that consist of the entire leaf may be treated with doses sufficient to achieve a 3.8-log₁₀ reduction of *L. monocytogenes* with no significant change in color or loss of texture. Endive preparations that are composed exclusively of the distal portion of the leaf, excluding the leaf midrib, may be treated with up to the current regulatory maximum dose (1.0 kGy, equivalent to a 4.8-log₁₀ reduction) without significant sensorial impact. This result highlights the key

role that the formulation of the finished product (e.g., a prepared salad mixture incorporating endive) plays in influencing the suitability of irradiation as a treatment for that product.

This study has shown that on a leafy green vegetable, *L. innocua* ATCC 51742 has a similar response to *L. monocytogenes* ATCC 49594 and may therefore be regarded as a valid surrogate model organism on this product for evaluations of radiation sensitivity. After very low radiation doses, equivalent to 2 D_{10} units, an initial decline in the *L. monocytogenes* population was fully recovered by 19 days in storage. Higher doses result in a more lasting suppression of *L. monocytogenes* on stored endive, and these doses (0.8 to 1.0 kGy) have little or no significant impact on the product's sensorial properties. In designing protocols that incorporate ionizing radiation in the processing of fresh vegetables, product formulation, e.g., proportion of leaf midrib material, will be a key factor in the successful implementation of this antimicrobial intervention.

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